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Chemical formation of hybrid di-nitrogen calls fungal codenitrification into question

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Chemical formation of hybrid di-nitrogen calls fungal codenitrification into question

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Removal of excess nitrogen (N) can best be achieved through denitrification processes that transform N in water and terrestrial ecosystems to di-nitrogen (N_2) gas. The greenhouse gas nitrous oxide (N_2O) is considered an intermediate or end-product in denitrification pathways. Both abiotic and biotic denitrification processes use a single N source to form N_2O. However, N_2 can be formed from two distinct N sources (known as hybrid N_2) through biologically mediated processes of anammox and codenitrification. We questioned if hybrid N_2 produced during fungal incubation at neutral pH could be attributed to abiotic nitrosation and if N_2O was consumed during N_2 formation. Experiments with gas chromatography indicated N_2 was formed in the presence of live and dead fungi and in the absence of fungi, while N_2O steadily increased. We used isotope pairing techniques and confirmed abiotic production of hybrid N_2 under both anoxic and 20% O_2 atmosphere conditions. Our findings question the assumptions that (1) N_2O is an intermediate required for N_2 formation, (2) production of N_2 and N_2O requires anaerobiosis, and (3) hybrid N_2 is evidence of codenitrification and/or anammox. The N cycle framework should include abiotic production of N_2.

The nitrogen (N) removal pathway known as denitrification is typically considered a biological process, where nitrate (NO_3^-) or nitrite (NO_2^-) is sequentially reduced by bacteria and archaea to nitric oxide (NO), nitrous oxide (N_2O) and finally inert di-nitrogen (N_2)[1]. Incomplete denitrification results in emission of N_2O, an important greenhouse gas now playing a primary role in stratospheric ozone depletion[2]. While prokaryotes are well known to denitrify[3], new findings indicate denitrification by eukaryotes, such as fungi, may be widespread[4–6]. Unlike prokaryotes, fungi do not have the genes encoding nitrous oxide reductase, which reduces N_2O to N_2, so fungal denitrification terminates at N_2O[7,8]. Denitrification occurs when a single N source is used to produce N_2O, such as NO_2^- or NO_3^-. Codenitrification occurs when individual atoms of the N_2O or N_2 molecules are derived from two distinct N sources[9,10], resulting in hybrid N_2[8]. Formation of hybrid N_2 is widely reported as evidence of anammox[11] or codenitrification[12–14]. Previously, isotope pairing experiments revealed chemodenitrification[15,16] and denitrification by the fungi *Bipolaris sorokiniana* used NO_2^- as the sole source for N_2O formation[16]. Fungal denitrification rates of NO_2^- to N_2O were similar under both anaerobic and microaerophilic conditions, contrary to classical denitrification, suggesting O_2 may not, in this case, be a strong regulator[16].

Incubation experiments with pure cultures and soils report a number of fungi may play a significant role in soil N trace gas production[5,6,17,18]. Fungi not only denitrify to N_2O but may also codenitrify to form N_2[8,9]. When O_2 is not available, some fungi (*Fusarium oxysporum*) reportedly co-metabolise organic forms of N to reduce NO_2^- or NO_3^- and form hybrid N_2[7,9,10]. This has been demonstrated in soils by tracing $^{29}N_2$ and $^{30}N_2$ following application of antibiotics to selectively inhibit bacteria or fungi[12,14]. Codenitrification is widely viewed as an anaerobic, enzymatically-mediated nitrosation process requiring low (<-1) formal oxidation state of the nucleophilic N[8]. Figure 1 illustrates how anammox, like codenitrification, also forms hybrid N_2, although anammox uses two forms of inorganic N, NO_2^- and ammonium (NH_4^+)[11,19].

The specific codenitrification pathway is unknown, but fungi reportedly reduce NO_2^- to NO using NO_2^- reductase (encoded by the *nirK* gene) and then reduce NO to N_2O using nitric oxide reductase (P450nor)[7]. The role of N_2O in the codenitrification process and in N_2 formation is not clear[20]. While utilisation of N_2O to form N_2

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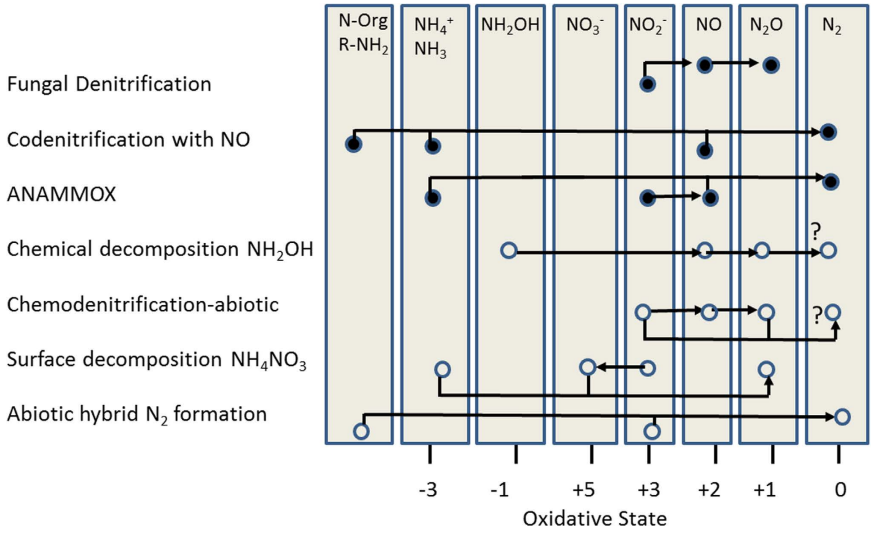

Figure 1. Schematic of codenitrification, anammox and known chemical denitrification pathways. Selected processes potentially leading to N_2O and N_2 formation, involved N compounds, their reaction pathways as well as their oxidation states are shown. Closed circles are biotic and open circles are abiotic reactions. Terms are defined as follows: Norg/R-NH_2, monomeric organically bound N forms; NH_4^+, ammonium; NH_3, ammonia; NH_2OH, hydroxylamine; NO_2^-, nitrite; NO_3^-, nitrate; NO, nitric oxide; N_2O, nitrous oxide; N_2, molecular dinitrogen. The last process, abiotic N_2 formation, was observed in this study. Figure 1 is a truncated adaptation from Butterbach-Bahl *et al.*[23], which is licensed under a Creative Commons Attribution License 3.0 (https://creativecommons.org/licenses/by/3.0/).

has been suggested as a plausible codenitrification pathway[8], reports of N_2O consumption during fungal production of N_2 (commonly observed during bacterial denitrification)[21] are lacking. Potentially bypassing reduction of N_2O to form N_2, in addition to formation of hybrid N_2, sets codenitrification and anammox apart from classical denitrification[22].

Laboratory studies commonly report evidence of fungal denitrification or codenitrification when pure cultures are incubated under anaerobic or microaerophilic conditions with sterile media, consisting of carbon, NO_2^- and mineral salts[5,6,9,10,16]. However, reduced metals in the medium, such as Fe(II), could provide electrons required for abiotic reduction of NO_2^- to N_2O, commonly known as chemodenitrification[15,23,24]. Chemodenitrification occurs through nitrosylation when reduced forms of inorganic N react with a metal centre to form N_2O in the absence of oxygen[15,24]. Nitrosylation may also drive abiotic formation of N_2, given high concentrations of metal and NO_2^-[25]. Wullstein and Gilmour[25] mixed 10,000 ppm N as potassium nitrite (KNO_2) with 5,000 ppm ferrous sulfate in an abiotic, anoxic reactor and recovered 15% of added N as N_2 within 3 d. In this case, the N_2 formed would have been denitrified from a single N source, KNO_2. Alternatively, chemical formation of hybrid N_2 through nitrosation is often ignored by biologists. Abiotic nitrosation of organic matter by NO_2^- in soil was first suggested by Nelson and Bremner[26] when they recovered over 20% of added N (5 mmols NO_2^- g^{-1} soil) as N_2 for sterile soil at neutral pH in helium (He) and heliox (20% O_2, 80% He) atmospheres. The isotopic composition of N_2 was not reported, but they indicated soil organic matter was an important factor[26]. Trimmer and Prudy[27] showed that deep seawater samples amended with $^{15}NO_2^-$ and $^{14}NH_4$ produced more $^{29}N_2$ when organic N (allylthiourea) was added. They suggested an alternative metabolic pathway to anammox but did not address the possibility of abiotic $^{29}N_2$ formation. Babbin *et al.*[28] also found organic N enhances anammox N_2 production. A common thread among chemical, anammox and fungal denitrification studies is NO_2^-. Thus, NO_2^- is a pivot-point for divergence in biological and chemical N trace gas production[29] (Fig. 1).

Here, we pursue open questions raised by this early work regarding abiotic N trace gas production recently reviewed by Heil *et al.*[30]. We aimed to investigate if previously unexplored sources of N_2O and N_2 could be contributing to reactive N removal and if N_2O was an intermediate in the abiotic N_2-production pathway. We questioned whether N_2 reportedly due to fungal codenitrification in pure culture experiments was formed abiotically in the presence and absence of O_2, given diverse inorganic and organic sources of N. New knowledge of abiotic N_2O and N_2 production would advance environmental N-removal research and applications and perhaps explain some mass balance discrepancies found in isotopic pairing studies[27,28].

To address these questions, we used a ubiquitous soil fungus, *Bipolaris sorokiniana* (Sacc.) Shoemaker [telemorph: *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur], as our model. Previously, we found that *B. sorokiniana* used NO_2^- as the sole source for denitrification to N_2O under anaerobic and microaerophilic conditions[16]. We also found NO_2^- as the sole source for chemodenitrification, which accounted for 6–8% of total N_2O production[16]. These results prompted further inquiry regarding fungal and chemical trace gas production of N_2. We aimed to more explicitly evaluate abiotic and biotic N_2O and N_2 production by comparing live fungi with fungal necromass incubated under strictly sterile conditions, with and without O_2. Pure culture experiments have demonstrated fungal and chemical N_2O production, but reports are lacking that indicate abiotic nitrosation of organic

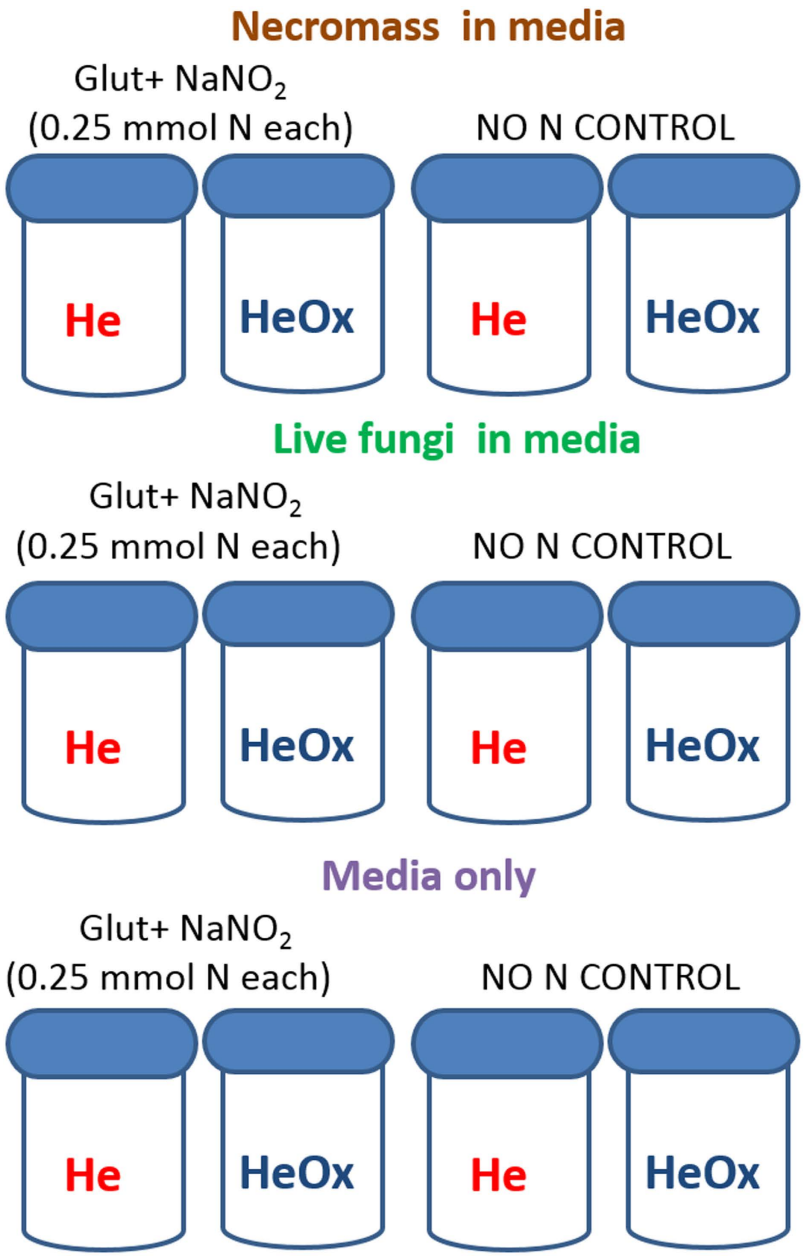

Figure 2. Experimental set-up to test how live fungi, necromass and media only incubated in an anoxic and 20% O_2 atmosphere affects production of N_2O, CO_2, O_2, and N_2 following addition of both organic and inorganic forms of N to pure cultures under aseptic conditions. Terms are defined as: Glut, glutamine; $NaNO_2$, sodium nitrite; He, helium; HeOx heliox.

compounds and formation of hybrid N_2. We include necromass in the design because amino acids and other nucleophilic compounds from necromass could potentially, in the absence of live fungi, react with NO^{2-} to form N_2. Further, necromass would provide more surface area for chemical decomposition of NO_2^- to N_2O[24] (Fig. 1). This would serve as a test for abiotic formation of N_2O and N_2 in the presence of decomposing organic material.

We aimed to assess biotic and abiotic N_2O and N_2 production using established microbiological laboratory incubation methods (Fig. 2). We exposed live fungi and necromass to both inorganic and organic N sources [0.25 mmol N as sodium nitrite ($NaNO_2$) and 0.25 mmol N as glutamine ($C_5H_{10}N_2O_3$); concentration of 20 mmol L^{-1} each] under two O_2 conditions (anaerobic and 20% O_2). For each treatment, there were replicate sets where N was not added to the media (No N control). We also included sterile media-only control vessels. Oxygen status was tightly controlled with airtight laboratory incubation vessels, where headspace was filled with either helium (anaerobic) or heliox (aerobic). Accumulation of headspace O_2, CO_2, N_2O, and N_2 were measured approximately every 6 h with a customised, robotic gas chromatography system[21]. Headspace O_2 was monitored to confirmed anaerobiosis was maintained during the 30 h incubation. Slopes of the linear increases in N_2O and N_2 in the headspace of each vessel were calculated to determine production rates. We used ANOVAs to test if

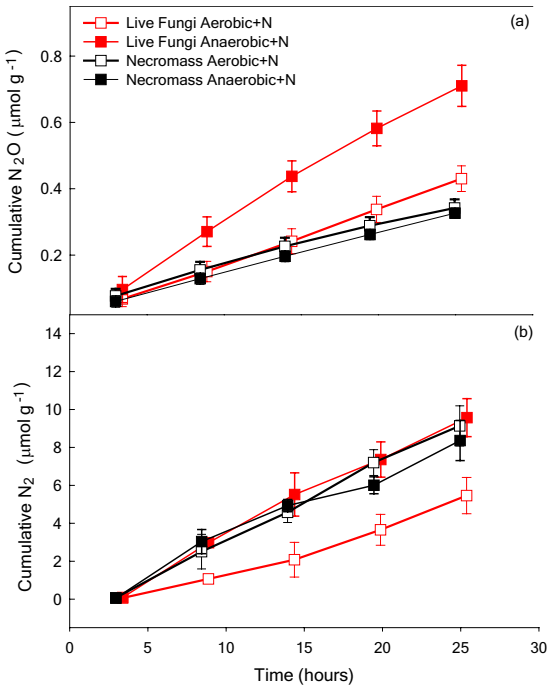

Figure 3. Kinetics of (**a**) N_2O and (**b**) N_2 production per g biomass over time following N addition at each time point as gases in the headspace accumulated. Average for each treatment are slightly staggered in time due to robotized measurement system, which is why comparisons were based on slopes over the entire incubation. Production rates of N_2O and N_2 were strongly affected by O_2 status [O_2 × fungal state (live or dead); $p < 0.001$] for those samples amended with N. Boxes represent average data; error bars, s.d.; n = 4.

production of N_2O and N_2 by live fungi was similar to necromass, and if both groups responded similarly to O_2. Production rates were calculated in units of μmol N as N_2O and N_2 g fungal biomass^{-1} h^{-1} and in units of μmol N as N_2O and N_2 h^{-1}. We performed a second incubation (illustration of this incubation design not shown) using isotope pairing techniques to determine if $^{29}N_2O$ or $^{30}N_2O$ were produced abiotically from sterile medium amended with glutamine and NO_2^- in the presence or absence of O_2. We used equal amounts of unlabelled glutamine and ^{15}N-labelled NO_2^- to achieve 0.5 mmol N and 1.0 mmol N, as well as a medium that was not amended with N.

Results and Discussion

We found both fungal states (live fungi and necromass) produced N_2O and N_2 but only for those samples amended with N. Linear production of N_2O and N_2, following N amendment, occurred quickly under completely anaerobic conditions and in a 20% O_2 atmosphere (Fig. 3). Average [(standard deviation (SD)] rate of N_2O produced by necromass was 0.012 (<0.001) μmol N_2O g biomass^{-1} h^{-1} under both aerobic and anaerobic conditions. Average N_2O production by live fungi was 0.017 (<0.001) μmol N_2O g biomass^{-1} h^{-1} and 0.028 (0.003) μmol N_2O g biomass^{-1} h^{-1}, respectively, under aerobic and anaerobic conditions (Fig. 3a). Rates of N_2O production were significantly greater for live fungi incubated anaerobically (O_2 × fungal state interaction; $p < 0.001$), indicating biological production of N_2O in the absence of O_2. In a 20% O_2 atmosphere, rates of N_2O production were similar to necromass. Our previous study indicated *B. sorokiniana* produced N_2O at similar rates when incubated anaerobically and in a 0.4% O_2 atmosphere[16]. Here, we used 20% O_2, and we found no evidence of microbial denitrification under these high-O_2 conditions.

Figure 4a provides a basis of comparison for N_2O recovered in the headspace of sterile media using comparable units (μmol N_2O h^{-1}). These data were not normalised per g of fungal biomass but illustrate accumulation of N_2O in sterile medium relative to necromass and live fungi. Nitrous oxide production rates were greater for necromass, as compared to media only (Fig. 4a). Necromass produced an average of 0.0034 μmol N_2O h^{-1} at both O_2 levels, as compared to 0.0011 μmol N_2O h^{-1} for sterile media. Greater N_2O for necromass suggests the presence of decaying biomass provided greater surface area for chemodenitrification[24] (Fig. 4a).

Rates of fungal N_2O production observed here are lower than other reports in the literature. Maeda *et al.*[6] found fungi carrying the *nirK* gene produced from 0.1 to 3.2 μmol N_2O g biomass^{-1} h^{-1}, and Rohe *et al.*[31] reported fungal N_2O production rates from 0.05 to 14 μmols N_2O h^{-1}. In both cases, denitrification varied with fungal species and N amendment. Here, *B. sorokiniana* does not carry the *nirK* gene, which may influence NO_2^- reduction to NO. Data on chemical formation of N_2O under oxic (20% O_2) conditions are lacking, so these first results challenge the paradigm that chemodenitrification requires anoxia[15]. Contrary to chemostat studies, we included fungal necromass, which contributed to abiotic N_2O. Results call into question if all N_2O produced in pure culture experiments[6,18,31] is enzymatically mediated if cultures include live and dead fungal or bacterial biomass.

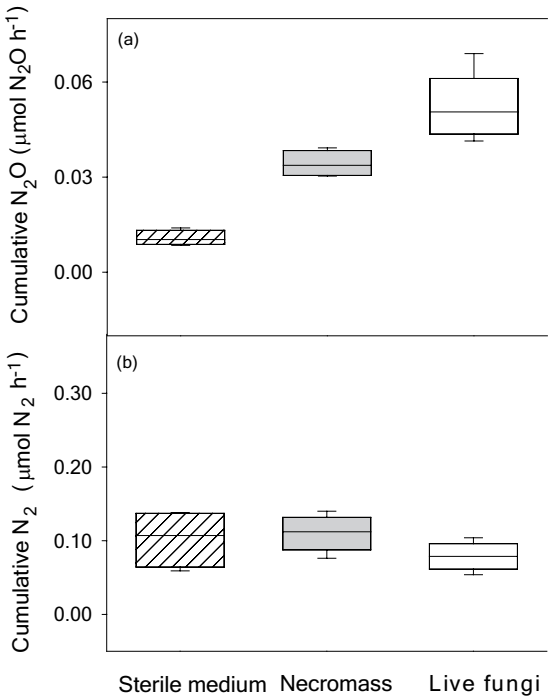

Figure 4. Abiotic and biotic contributions to rates of (**a**) N_2O and (**b**) N_2 accumulated per hour following N addition under both aerobic and anaerobic conditions for sterile medium, necromass and live fungi. Boxes represent the 90th percentile data; error bars, s.d.; n = 4. Median values are the lines horizontally bisecting each box.

We found no evidence of biological N_2 production in the presence or absence of necromass (Fig. 3b). Lowest rates of N_2 production were observed for live fungi incubated in a 20% O_2 atmosphere ($O_2 \times$ fungal state interaction; $p < 0.001$). Rates of N_2 produced in the headspace of live fungi were 0.243 (0.039) μmol N_2 g biomass^{-1} h^{-1} and 0.424 (0.049) μmol N_2 g biomass^{-1} h^{-1} under aerobic and anaerobic conditions, respectively. Rates of N_2 produced in the headspace of necromass were 0.415 (0.041) μmol N_2 g biomass^{-1} h^{-1} and 0.356 (0.047) μmol N_2 g biomass^{-1} h^{-1} under aerobic and anaerobic conditions, respectively. Our results indicate there is a strong abiotic component to measured rates of N_2 production and challenge the assumption that fungal N_2 production[7] requires anoxic or microaerophilic conditions[8].

Most of the N_2 accumulated in the headspace for live *B. sorokiniana* was commensurately found in the headspace of *B. sorokiniana* necromass (Fig. 3b), which points to chemical N_2 formation. We show chemical production of N_2 (μmol h^{-1}) for sterile medium relative to necromass and live fungi at both O_2 levels in Fig. 4b. Overall average rates of N_2 formation were 0.12 (0.039) μmol N_2 h^{-1} for sterile medium, 0.11 (0.024) μmol N_2 h^{-1} for necromass, and 0.08 (0.018) μmol N_2 h^{-1} for live fungi. Others have reported much higher rates of anoxic chemical N_2 formation (7–32 μmol N_2 h^{-1}) at neutral pH when high concentrations of reduced metals were reacted with high concentrations of NO_2^- [25] and when high NO_2^- solutions (20 M) were added to autoclaved soil[26], but these did not report if hybrid N_2 was formed or not. Comparable rates of abiotic N_2 production for necromass and sterile medium caused us to question if abiotic N_2, like N_2O, could result from nitrosylation of inorganic N only or from nitrosation to form hybrid N_2. Organic N has been found to increase biological production of $^{29}N_2$ in oceanic studies[32], but our results (Fig. 4b) indicated there may also be abiotic processes that contribute strongly to $^{29}N_2$ production. Consequently, we determined if both inorganic and organic N were used to produce N_2 abiotically in a separate, isotope pairing experiment.

The headspace above sterile media incubated under oxic and anoxic conditions with ^{15}N-labelled $NaNO_2$ and unlabelled $C_5H_{10}N_2O_3$ indicated abiotic, formation of hybrid N_2. Almost all (99.9%) of the N_2 produced was $^{29}N_2$ (Table 1). The $^{29}N_2$ production was proportional to the mass of added N and approximately 3–4% of the total N added was transformed to $^{29}N_2$ under both oxic and anoxic conditions and at both levels of NO_2^- addition. Results demonstrate hybrid formation of N_2 is not necessarily enzymatically mediated and does not required anoxia. Formation of abiotic, hybrid N_2 by two distinct inorganic N molecules has not been ruled out here. Previous bodies of work that use formation of $^{29}N_2$ as evidence of anammox and/or codenitrification[12,14,32,33] need to be reviewed with respect to abiotic N_2 production.

Linear increases in cumulative N_2O and N_2 over time under aerobic and anaerobic conditions for both live fungi and necromass are shown in Fig. 3. These data suggest that N_2O was not consumed during the incubation and both gases were produced independently. This finding contrasts with bacterial denitrification[22], where a sharp rise in microbial production of N_2O is followed by a rise in biological reduction of N_2O to N_2, and a drop in cumulative N_2O production[21]. *Bipolaris sorokiniana* denitrifies NO_2^- only and does not use glutamine to form N_2O[16]. If the pathway to N_2 were through the intermediate N_2O, we would expect not only N_2O consumption but

N addition (mmol N)	$^{29}N_2$ (^{14}N,^{15}N) (μmol) aerobic	$^{30}N_2$ (^{15}N,^{15}N) (μmol) aerobic	$^{29}N_2$ (^{14}N,^{15}N) (μmol) anaerobic	$^{30}N_2$ (^{15}N,^{15}N) (μmol) anaerobic
0	−0.012 (<0.001)	0.000 (0.000)	−0.005 (0.005)	<0.001 (<0.001)
0.5	16.274 (2.045)	0.001 (<0.001)	15.759 (0.995)	0.003 (0.002)
1.0	40.289 (2.318)	0.002 (<0.001)	31.025 (3.144)	0.007 (0.001)

Table 1. Average (SD) of $^{29}N_2$ and $^{30}N_2$ recovered in the headspace following aerobic and anaerobic incubation of sterile media at three levels of N addition, where added N comprised 50% N from unlabelled $C_5H_{10}N_2O_3$ and 50% N from labelled ^{15}N-$NaNO_2$ (n = 5). Samples were incubated for >7 d prior to isotopic analyses. Values were adjusted according to helium or heliox blanks.

also accumulation of $^{30}N_2$ in the isotopic pairing experiment. Like anammox, our abiotic N_2 production results indicate N_2O formation was bypassed in the pathway to N_2 (Fig. 1).

We show compelling evidence that formation of $^{29}N_2$ does not result from solely biotic nitrosation but also abiotic nitrosation. Abiotic nitrosation occurred both in the presence and absence of O_2, and abiotic N_2 was formed exclusively through hybridisation of inorganic and organic N sources and did not require the N_2O intermediary. Experimental protocols, including N concentrations, were in accordance with fungal denitrification[6,16] and codenitrification[10] studies but in the absence of soils and sediments. In soil, NO^{2-} accumulates when excess free NH_3 inhibits bacterial NO_2^- oxidation, which is often a consequence of urea hydrolysis[30]. Venterea *et al.*[34] recovered 3–60% of added urea N as NO_2^-, suggesting high soil NO^{2-} is likely following urea addition (depending upon conditions and application rate). Based on these data[34], a 1-kg bovine urine addition to soil (N concentration of 0.4 mmol kg^{-1})[35] could result in up to 0.24 mmol NO_2^-. Here, we added 0.25 mmol NO_2^- to 10 ml of fungal culture, which is on the high end of this scale. While we would expect abiotic formation of hybrid N_2 via nitrosation of organic N to be more likely in grazed or fertilised agroecosystems, further N_2 investigations with soil and at lower NO_2^- levels are needed to bridge the gap between pure culture and environmental applications.

Other areas where NO_2^- could accumulate include laboratory incubations where antibiotic inhibitors are added to soil and used to partition N_2 and N_2O production into fungal and bacterial contributions[12,14]. These data may be subject to artefacts if antibiotics repress NO_2^- oxidation, leading to NO_2^- accumulation and potential abiotic formation of N_2 and/or N_2O. Our report also calls into question if codenitrification alone accounts for N_2 and N_2O emissions following high doses of N as urea (approximately 9 mmol g^{-1} soil)[13] and/or NO_2^- (166 mmol L^{-1})[10]. If abiotic, hybrid N_2 is formed under these conditions, our understanding of soil fungal codenitrification and N trace gas emissions would need to be re-examined. We offer new insight into chemodenitrification of NO_2^- to N_2 and present an alternative 'N_2O bypass pathway'. Finally, we conclude the potential for aerobic, abiotic removal of excess reactive N in terrestrial and aquatic ecosystems presents environmental mitigation research opportunities, particularly where transitory or chronic NO_2^- accumulation occurs.

Methods

Fungal incubations were conducted as described in Phillips *et al.* (2016) with the same culture ICMP 6809 isolated as a pathogen of *Hordeum distichon* in New Zealand. (https://scd.landcareresearch.co.nz/Specimen/ICMP_6809) GenBank: KU194490. We used laboratory incubations and controlled O_2 status to evaluate if rates of N_2O, N_2 and CO_2 production rates vary with aerobic conditions, as commonly reported for bacteria[20]. Experimental design was similar to previous work[16], with 4 replicates for each treatment (N with and without O_2; no N with or without O_2). Cultures were grown in medium similar to other fungal denitrification studies[9,10,31], consisting of 1% glucose, 0.2% peptone and inorganic salts[5,36]. The only N source in this medium used for fungal growth was peptone. One day prior to the experiment, the growth medium was washed off cultures and replaced with the same medium that was identical except it was free of peptone and so contained no N. Using the peptone-free medium herein, two amendment solutions were prepared for fungal inoculation: (a) media without N and (b) 0.25 mmol N as $NaNO_2$ and 0.25 mmol N as $C_5H_{10}N_2O_3$. Nitrogen concentration was 0.04 mmol N L^{-1}. Necromass was obtained by heating live *B. sorokiniana* at 60 °C for 48 h. Cell death was confirmed by (a) microscopy and (b) lack of CO_2 respiration over a 24 h period under aerobic and anaerobic conditions. Approximately 10 ml live fungi or fungal necromass were blindly pipetted by a second independent scientist into 0.125 L serum bottles that were then amended with either media that included 0.25 mmol N as $NaNO_2$ and 0.25 mmol N as $C_5H_{10}N_2O_3$ or media without N and mixed gently. Additional bottles containing sterile media solutions (with and without N) only and without fungi were also prepared. Oxygen status was controlled with airtight laboratory incubation vessels for all necromass, live fungi and media only samples. Headspace of each sample was evacuated and filled with either He (anaerobic) or heliox [aerobic (80% He, 20% O_2)] within 2 hr following inoculation[18,20]. Headspace gases were quantified approximately every 6 h at 19 °C using a robotic gas chromatograph (GC) fitted with electron capture and thermal conductivity detectors according to McMillan *et al.*[21]. Helium or heliox blank standards were included in each GC run. Cumulative production rates were calculated as slopes of the masses (μmol) of N_2O or N_2 measured over time (h) per g of fungal biomass. Vessels incubated in He remained anoxic with the exception of necromass, where we observed 0.05% O_2. Vessels incubated in heliox remained above 19% O_2 with the exception of live fungi incubated without N, where 5% of the headspace O_2 was consumed. As reported previously, addition of NO_2^- inhibited O_2 consumption and CO_2 respiration by live fungi[16]. Fungal biomass was determined as the difference in mass with and without media after air-drying each vessel post-incubation[16]. For comparisons with sterile media, rates were also calculated as slopes of the masses (μmol) of N_2O and N_2 measured over time. Sterility was maintained and conditions remained constant, including pH (6.2–6.9). Sterility was tested at the end of gas sampling by pipetting 200 μL medium used in the experiment onto blood agar plates and incubating

under aerobic, anaerobic (AnaeroGen™, Thermo Scientific), and enriched CO_2 (3.5–9%; CO_2Gen™, Thermo Scientific) conditions. Further, medium was also pipetted onto yeast nutrient agar, brain heart infusion agar, and potato dextrose agar plates under aerobic conditions. No evidence of fungal or bacterial growth was observed following 3, 7, and 10 d incubations at 26 °C. Data were analysed to test for effects of O_2 and live fungi on either N_2O or N_2 production rates with a generalised linear model. We analysed only those samples amended with N because samples without N did not produce N_2O or N_2. Log transformations were employed when data did not meet the assumptions of normality. Treatments variances met assumptions of homoscedasticity. All interactions were tested and remained in the model if significant.

Di-nitrogen isotopes were evaluated in a separate experiment to assess sources of N used in abiotic production of N_2 by adding $Na^{15}NO_2$ and $C_5H_{10}N_2O_3$ to sterile medium at neutral pH, as described above. We aimed to determine if N_2 would be produced through combination of $^{15}NO_2$ only (thus forming $^{30}N_2$) or through combination of both $C_5H_{10}N_2O_3$ and $^{15}NO_2$ (thus forming the hybrid $^{29}N_2$) for N-enriched medium relative to no-N medium only. In this experiment, we used five replicates at three levels of N: (a) no-N, (b) 0.25 mmol $NaNO_2$ and 0.25 mmol $C_5H_{10}N_2O_3$, and (c) 0.5 mmol $NaNO_2$ and 0.5 mmol $C_5H_{10}N_2O_3$. We used the same concentration of N in each N treatment but doubled the mass of N added. Vials were prepared under aerobic and anaerobic conditions as described previously and accompanied by He blanks. The aerobic experiment was conducted separately from the anaerobic experiment, which obviated testing for effects of O_2 on masses of $^{29}N_2$ and $^{30}N_2$. Headspace $^{29}N_2$ and $^{30}N_2$ were measured on a continuous-flow isotope ratio mass spectrometer (Thermo Finnigan Delta V, Thermo Scientific) in line with an automated gas bench interface (Thermo Gas Bench II). Precision of the isotopic analysis was <0.001atom%.

References

1. Zumft, W. G. Cell biology and molecular basis of denitrification. *Microbiol. Molec. Biol. Rev.* **61,** 533–616 (1997).
2. Ravishankara, A. R., Daniel, J. S. & Portmann, R. W. Nitrous oxide (N_2O): The dominant ozone-depleting substance emitted in the 21st century. *Science* **326,** 123–125; doi: 10.1126/science.1176985 (2009).
3. Philippot, L., Hallin, S. & Schloter, M. Ecology of denitrifying prokaryotes in agricultural soil. *Adv. Agron.* **96,** 249; doi: 10.1016/S0065-2113(07)96003-4 (2007).
4. Yang, H., Gandhi, H., Ostrom, N. E. & Hegg, E. L. Isotopic fractionation by a fungal P450 nitric oxide reductase during the production of N_2O. *Environ. Sci.Tech.* **48,** 10707–10715; doi: 10.1021/es501912d (2014).
5. Rohe, L. *et al.* Fungal oxygen exchange between denitrification intermediates and water. *Rapid Comm. Mass Spectrom.* **28,** 377–384; doi: 10.1002/rcm.6790 (2014).
6. Maeda, K. *et al.* N_2O production, a widespread trait in fungi. *Sci. Rep.* **5,** 9697; doi: 10.1038/srep09697 (2015).
7. Shoun, H., Fushinobu, S., Jiang, L., Kim, S. & Wakagi, T. Fungal denitrification and nitric oxide reductase cytochrome P450nor. *Philosoph. Trans. Royal Soc. B: Biol. Sci.* **367,** 1186–1194; doi: 10.1098/rstb.2011.0335 (2012).
8. Spott, O., Russow, R. & Stange, C. F. Formation of hybrid N_2O and hybrid N_2 due to codenitrification: First review of a barely considered process of microbially mediated N-nitrosation. *Soil Biology and Biochemistry* **43,** 1995–2011; doi: 10.1016/j.soilbio.2011.06.014 (2011).
9. Tanimoto, T., Hatano, K.-i., Kim, D.-h., Uchiyama, H. & Shoun, H. Co-denitrification by the denitrifying system of the fungus *Fusarium oxysporum*. *FEMS Microbiol. Lett.* **93,** 177–180; doi: 10.1111/j.1574-6968.1992.tb05086.x (1992).
10. Shoun, H., Kim, D.-H., Uchiyama, H. & Sugiyama, J. Denitrification by fungi. *FEMS Microbiology Letters* **94,** 277–281; doi: 10.1111/j.1574-6968.1992.tb05331.x (1992).
11. Kuypers, M. M. M. *et al.* Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium oxidation. *Proc. Nat. Acad. Sci., USA* **102,** 6478–6483; doi: 10.1073/pnas.0502088102 (2005).
12. Long, A., Heitman, J., Tobias, C., Phillips, R. & Song, B. Co-occurring anammox, denitrification, and codenitrification in agricultural soils. *Appl. Environ. Microbiol.* **79,** 168–176; doi: 10.1128/aem.02520-12 (2013).
13. Selbie, D. R. *et al.* Confirmation of co-denitrification in grazed grassland. *Sci. Rep.* **5,** 17361; doi: 10.1038/srep17361 (2015).
14. Laughlin, R. J. & Stevens, R. J. Evidence for fungal dominance of denitrification and codenitrification in a grassland soil. *Soil Sci. Soc. Amer. J.* **66,** 1540–1548 (2002).
15. Jones, L. C., Peters, B., Lezama Pacheco, J. S., Casciotti, K. L. & Fendorf, S. Stable isotopes and iron oxide mineral products as markers of chemodenitrification. *Environ. Sci. Tech.* **49,** 3444–3452; doi: 10.1021/es504862x (2015).
16. Phillips, R. L. *et al.* Fungal denitrification: *Bipolaris sorokiniana* exclusively denitrifies inorganic nitrogen in the presence and absence of oxygen. *FEMS MIcrobiol. Lett.* **363**; doi: 10.1093/femsle/fnw007 (2016).
17. Sutka, R. L., Adams, G. C., Ostrom, N. E. & Ostrom, P. H. Isotopologue fractionation during N_2O production by fungal denitrification. *Rapid Comm. Mass Spectrom.* **22,** 3989–3996; doi: 10.1002/rcm.3820 (2008).
18. Zhou, Z., Takaya, N., Sakairi, M. A. C. & Shoun, H. Oxygen requirement for denitrification by the fungus *Fusarium oxysporum*. *Arch. Microbiol.* **175,** 19–25; doi: 10.1007/s002030000231 (2001).
19. Schmid, M. C. *et al.* Anaerobic ammonium-oxidizing bacteria in marine environments: Widespread occurrence but low diversity. *Environ. Microbiol.* **9,** 1476–1484 (2007).
20. Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R. & Zechmeister-Boltenstern, S. Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Phil. Trans.Royal Soc. B: Biol. Sci.* **368,** 20120122; doi: 10.1098/rstb.2013.0122 (2013).
21. McMillan, A. M. S. *et al.* Can pH amendments in grazed pastures help reduce N_2O emissions from denitrification? – The effects of liming and urine addition on the completion of denitrification in fluvial and volcanic soils. *Soil Biol. Biochem.* **93,** 90–104; doi: http://dx.doi.org/10.1016/j.soilbio.2015.10.013 (2016).
22. Firestone, M. K. & Tiedje, J. M. Temporal change in nitrous oxide and dinitrogen from denitrification following onset of anaerobiosis. *Appl. Environ. Microbiol.* **38,** 673–679; doi: http://aem.asm.org/content/38/4/673 (1979).
23. Moraghan, J. & Buresh, R. Chemical reduction of nitrite and nitrous oxide by ferrous iron. *Soil Sci. Soc. Amer. J.* **41,** 47–50 (1977).
24. Kampschreur, M. J. *et al.* Emission of nitrous oxide and nitric oxide from a full-scale single-stage nitritation-anammox reactor. *Water Sci. Tech.* **60,** 3211–3217 (2009).
25. Wullstein, L. H. & Gilmour, C. M. Non-enzymatic formation of nitrogen gas. *Nature* **210,** 1150–1151; doi: 10.1038/2101150a0 (1966).
26. Nelson, D. W. & Bremner, J. M. Gaseous products of nitrite decomposition in soils. *Soil Biol. Biochem.* **2,** 203–IN208; doi: 10.1016/0038-0717(70)90008-8 (1970).
27. Trimmer, M. & Purdy, K. J. Evidence for the direct oxidation of organic nitrogen to N_2 gas in the Arabian Sea. *ISME J.* **6,** 1798–1800; doi: 10.1038/ismej2012.18 (2012).
28. Babbin, A. R., Keil, R. G., Devol, A. H. & Ward, B. B. Organic matter stoichiometry, flux and oxygen control nitrogen loss in the ocean. *Science* **344,** 406–408; doi: 10.1126/science.1248364 (2014).

29. Venterea, R. T. Nitrite-driven nitrous oxide production under aerobic soil conditions: kinetics and biochemical controls. *Global Change Biology* **13,** 1–12; doi: 10.1111/j.1365-2486.2007.01389.x (2007).
30. Heil, J., Vereecken, H. & Bruggemann, N. A review of chemical reactions of nitrification intermediates and their role in nitrogen cycling and nitrogen trace gas formation in soil. *Europ. J. Soil Sci.* **67,** 23–39; doi: 10.1111/ejss.12306 (2016).
31. Rohe, L. *et al.* Dual isotope and isotopomer signatures of nitrous oxide from fungal denitrification – a pure culture study. *Rapid Comm. Mass Spectrom.* **28,** 1893–1903; doi: 10.1002/rcm.6975 (2014).
32. Zhu, G. *et al.* Occurrence, activity and contribution of anammox in some freshwater extreme environments. *Environ. Microbiol. Rep.* (2015).
33. Ward, B. B. *et al.* Denitrification as the dominant nitrogen loss process in the Arabian Sea. *Nature* **461,** 78–81; doi: 10.1038/nature08276 (2009).
34. Venterea, R. T. *et al.* Ammonium sorption and ammonia inhibition of nitrite-oxidizing bacteria explain contrasting soil N_2O production. *Scientific Reports* **5,** 12153, doi: 10.1038/srep12153; http://www.nature.com/articles/srep12153#supplementary-information (2015).
35. Hoogendoorn, C. J., Betteridge, K., Costall, D. A. & Ledgard, S. F. Nitrogen concentration in the urine of cattle, sheep and deer grazing a common ryegrass/cocksfoot/white clover pasture. *New Zealand J. Agri. Res.* **53,** 235–243; doi: 10.1080/00288233.2010.499899 (2010).
36. Marx, D. H. & Bryan, W. C. Growth and ectomycorrhizal development of loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. *For. Sci.* **21,** 245–254; doi: 10.1080/00288233.2010.499899 (1975).

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Author Contributions

R.L.P., B.S. and G.G. designed the experiments, T.P. performed lab experiments, A.M.S.M. analysed chromatographic data, C.T. performed isotope pairing experiments, B.S.W. and G.G. cultured, and verified fungal isolates, and R.L.P. and B.S. wrote the manuscript.

Additional Information

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